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13. ABSTRACT Several tumor suppressor genes (TSGs) have been cloned and found to be mutated in a variety of cancers, including breast cancer. However, few breast cancer-specific TSGs are known. The purposes of this proposal are to (1) generate cDNA expression libraries from reduction mammoplasties, (2) use a novel functional assay to clone new TSGs specific to human breast cancer, and (3) identify their characteristics, regulation and function. We are utilizing the tetracycline (tet) regulable system. We have constructed a cDNA library from normal human breast epithelia and cloned this cDNA library into a vector that is negatively regulated by tet repressor (tetR) and simultaneously expresses the enhanced green fluorescent protein. These vectors were then co-transfected into LCC6, MDA231, and MCF-7 cells that have the capability to express tetR. Upon withdrawal of tet, the repressed expression of the cDNA of interest is released, and the cDNA is expressed. Using a novel dye retained in nonproliferating cells, we were able to identify growth inhibited clones which were then sorted by Flow Cytometry. This functional screen has provided the basis for identifying TSGs that are expressed in the growth inhibited cells. Using PCR, we have obtained and sequenced two insert sequences. One is a putative TSG on chromosome #9 (725bp) but the other sequence is vector DNA.

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3. Table of Contents

4. Introduction

Breast cancer is one of the most common malignancies of women in the United States. Most molecular genetic abnormalities contributing to breast cancer susceptibility remain unknown. Recent studies have revealed genomic changes in breast cancer, including amplification of proto-oncogenes, such as c-myc, c-erbB2, int-2, bcl-1, PRAD-1, EMS, EGF receptor (c-erbB 1), IGF-1 receptor, flg and bek (1,2) and intragenic mutations or suppressed expression in tumor suppressor genes (TSGs) including p53, Rb and p33 (3-5). TSGs constitute a relatively new class of genes and has been implicated in regulation of cell proliferation, cell cycle progression, apoptosis induction, and DNA repair and recombination. Many TSGs have been cloned from humans and found to be mutated in variety of human cancers (3-13). The study of TSGs should not only speed up basic cancer research, but it may also aid in the early diagnosis, prognostication, and treatment of human malignancies. Loss of heterozygosity (LOH), which is usually considered the hallmark of TSGs, has been observed in at least 15 out of the 23 pairs of chromosomes in human tumors. This result suggests that there may be numerous TSGs. However, only two genes specifically related to breast cancer have so far been cloned (BRCA-1) (14-16) or mapped to a specific chromosomal segment (BRCA-2) (17, 18); moreover the prevalence of intragenic somatic mutations in BRCA-1 is not very high in sporadic breast tumors (less than 10% of cases). Therefore, it seems likely that the cloning of new tumor suppressor genes of specific importance in breast cancer will be important and promising task for future research into this common disease. Putative TSGs that are growth-suppressive and specifically altered in breast cancers may be useful tools for the early diagnosis, prognostication, and eventual treatment of human breast cancers.

5 Body of Report

A. Brief statement of ideas and reasoning

Tumor suppressor genes (TSGs) function in normal tissues by regulating the growth of normal cells. Mutations, deletions, or other modes of inactivation of TSGs should contribute to uncontrolled growth and malignant transformation of normal cells. Many TSGs have been cloned from humans and found mutated in variety of human cancers, including breast cancer (3-18). Many human chromosomes show high rates of loss of heterozygosity (LOH) in breast cancer. However, relatively few breast cancer-specific TSGs, such as BRCA-1, have been cloned (14). The mutation rate of BRCA-1 in primary human breast tumors is less than 10% (15). Therefore, additional specific tumor suppressor genes for breast cancer are likely to exist. A cDNA expression library made from mRNA of normal human mammary glands should contain potential TSGs for human breast cancer, and thus can reasonably be used to isolate TSG(s) specific to breast cancer that inhibit the growth of breast cancer cells. We propose a functional screen for

the discovery of TSGs, which dramatically decreases the time to isolation and *a priori* demonstrates the function of novel TSGs. In addition, by using a cDNA expression library from normal human breast epithelia, transfected into breast cancer cells, we hope to clone TSG(s) that are specific to breast cancer.

B. Hypotheses/Purposes

We hypothesize that:

1) Normal human mammary gland epithelia should contain all normally expressed potential TSGs for breast cancer.

2) TSGs play an important role in growth regulation of breast cancer cells in culture.

3) TSGs contribute significantly to the carcinogenic process in a significant portion of breast cancers.

The purpose of this proposal is to clone TSG(s) specific to breast cancer, examine their alteration in primary breast tumors, identify their characteristics, and ultimately study their regulation and function.

C. Technical Objectives

1). Construct a cDNA expression library made from normal human mammary gland epithelia (completed).

2) Transduce cells with the expression library and select clonal populations of growth inhibited cells (completed).

3). Characterize the cloned TSGs by sequence homology analysis and study their functional effect on *in vitro* tumorigenesis for the most promising candidates (partially completed).

4). In the long term: to study the regulation of cloned TSGs by finding their promoter regions and regulatory elements.

D. Experimental Methods (Overview)

Outline and rationale for approach

A major problem in the identification of growth inhibitory genes in a functional assay is that it is the non-proliferating (suppressed) cells that are the cells containing the genes of interest. We have constructed a novel approach that we believe is optimized for the specific purpose of identifying growth suppressor genes. Thus, we have utilized the tetracycline repressor (tetR)-based gene expression system. We have directionally cloned the cDNA library (see below) into an expression vector placing each cDNAs under the control of the tetracycline resistance operon that is regulated by tetR (19-22). These vectors were also able to express enhanced green fluorescence protein (EGFP) reporter by which the expression of genes of interest were monitored indirectly (22). These plasmids were co-transfected with the puromycin resistant plasmid into MCF-7 cells, MDA435/LCC6 cells and MDA-MB-231 cells already transfected with a plasmid expressing both the tetR repressor and the G418 resistance marker (MCF-$7^{tetR+neoR}$, $LCC6^{tetR+neoR}$, $231^{tetR+neoR}$). Upon withdrawal of doxycycline, the tetR/VP16 binds and activates transcription of the cDNA (19, 21). Double resistant and EGFP expressing cells were selected and expression of the gene of interest studied in the presence of increasing concentrations of doxycycline.

While we have a method to regulate genes expression, we also have an approach for enriching bulk transfected cell populations for growth inhibited cells. We have used our adaptation of the dye enrichment method of Maines et al. (23). The dye, PKH26-GL, (Sigma Chemical Co. St Louis, MO) (24) is non-toxic and specifically retained in non-proliferating cells. Since Flow Cytometry can be used to visually sort cells retaining dye (cells are maintained in the absence of tet), and there is a state-of-the-art Flow Cytometry Core Facility at the Lombardi Cancer Center, we were able to rapidly enrich the population for the growth inhibited cells, including cells that are completely growth arrested, sorting for the most red fluorescent cells (23,24). Thus, following the 24 hr recovery period immediately post-transfection, the cells were selected with puromycin, the resistance marker coexpressed in the plasmids containing the cDNA library. The concentration of puromycin was 1 μg/ml.

Surviving cell populations were stained with PKH26-GL and grown, now in the absence of tet, for the equivalent of several generations as described by Maines *et al.* (23). The estimated generation time for non-inhibited MCF-7 cells is 24-36 hrs and LCC6 and MDA231 18-24 hrs (25). Subsequently, single cells were aseptically sorted by Flow Cytometry (double sort - red for growth inhibition, green for gene expression) into the wells of 96-well plates. This provided individual cell clones expressing putative growth inhibitory genes. Cell clones containing growth suppressing cDNAs were then rapidly expanded by adding doxycycline to block the putative TSG expression and release its

growth suppression. The putative growth inhibitory genes were analyzed by PCR, subcloning and sequencing. Growth suppressor activity can be further confirmed, in a functional assay, by following experiments: (1) cloning putative tumor suppressing genes into expression vector; (2) transiently transfecting MCF-7 cells, LCC6 cells, and MDA231 cells with these vectors containing the genes interested; (3) observing cell growth by cell-cycle analysis using Flow Cytometry. RNA containing the expressed putative suppressor genes can be obtained, by introducing different concentrations of doxycyclineto the culture medium to establish a tet-based dose response relationship for cell proliferation.

Using this approach:

(1) Growth inhibition is apparent only upon removal of tet and this reduces the background due to insertional mutagenesis that could randomly produce slowly proliferating/growth inhibited cells independent of the inserted cDNA.

(2) We can identify genes that completely suppress proliferation, as well as genes that merely reduce the rate of proliferation.

(3) We can rapidly identify, in a functional assay, genes that specifically inhibit estrogen-regulated growth.

E. Progress on Technical Objectives from SOW.

While we have not completed all the work as hoped, we have completed a substantial proportion of the proposed studies. In addition, as a training grant, the Fellow who completed most of the studies (Dr. Pu) successfully entered a Residency training program. Dr. Zhu, who used the few remaining resources during the no-cost extension, continued the training and the last few months of work associated with this award. The training included active participation in regular laboratory and Cancer Center data meetings, Cancer Center seminar series, and auditing several courses. For example, Dr. Zhu recently completed a course on statistics and a course on bioinformatics, to learn how to integrate the data from these types of studies with those available on the internet. Completion of the technical (scientific) aspects of this award are detailed below.

Title: Tumor Suppressor Genes in Breast Cancer
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Technical Objective 1: Identify putative TSG(s)

Task 1: Construct and Characterize cDNA library.

Task 2: Re-construct plasmid cDNA library and transfect MCF-7$^{tetR+neoR}$ cells, LCC6$^{tetR+neoR}$cells, and MDA231$^{tetR+neoR}$ cells.

Task 3/4: Identify cells containing growth inhibitory genes and Clone TSG(s).

Construction of a cDNA expression library from normal human breast epithelia

To successfully isolate mRNA from normal human breast tissues, there are three important concerns: 1) effective disruption of tissue and denaturation of nucleoprotein complexes, 2) inactivation of RNase activity, and 3) purification of mRNA away from contaminating DNA and protein. Thus, we used the PolyA TractR system 1000 (Promega Corp. Cat.#Z5410), since this procedure yields an essentially pure fraction of mature mRNA without extractions or precipitations. This method combines guanidine thiocyanate (GTC) and β-Mercaptoethanol to inactivate RNase. Then GTC is associated with SDS to disrupt nucleoproteins and allows for hybridization between the poly(A) sequence of mRNAs and a synthetic biotinylated oligo(dT)probe. The biotinylated oligo(dT):mRNA hybrids were captured with Streptavidin Paramagnetic Particles (SA-PMPs). The particles were washed at high stringency and purified mRNA was eluted by the addition of nuclease-free deionized water (30).

To construct a cDNA library, we used The CapFinderTM PCR cDNA Library Construction Kit (CLONTECH Laboratories, Inc., Cat.#K1051-1). This is a novel, PCR-based method for making high-quality libraries from a small quantity of RNA. This technique also utilizes the unique CapSwitchTM oligonucleotide in the first-strand synthesis, followed by long distance PCR amplification to produce high yields of full-length, double-strand (ds) cDNA (31-33). Therefore, we performed the reverse transcription (RT) to transcribe 100 ng poly A+ mRNA into single-strand (ss) DNA by using reverse transcriptase, a modified oligo (dT) primer (CDS/3' PCR primer) (32) and a CapSwitch oligonucleotide. The CDS/3' PCR primer was used to prime the first-strand reaction. The CapSwitch oligonucleotide was used as a short, extended template at the 5' end for the RT. When the RT reached the 5' end of the mRNA, the enzyme switched templates and continued replicating to the end of the CapSwitch oligonucleotide. The resulting full-length ss cDNA contained the complete 5' end of the mRNA and the sequence complementary to the CapSwitch oligonucleotide, which then served as a PCR priming site (CapSwitch anchor). The PCR was performed by directly using the CapSwitch anchor. In this reaction, only those oligo (dT)-primed ss cDNA having a CapSwitch anchor sequence at the 5' end served as templates and was amplified using the 3' and 5' PCR primers and Advantage KlenTaq Polymerase (33). This selective

amplification did not allow incomplete cDNAs and cDNA transcribed from polyA-RNA to be amplified, therefore eliminating library contamination by genomic and polyA-RNA.

The ds cDNAs were ligated using T4 ligase to specific-adaptors which contains a pre-existing EcoRI "sticky end" and phosphorylated blunt end for the efficient ligation to the blunt-ended cDNA. This ligation eliminated the need to methylate and EcoRI-digest the cDNA, and left the internal EcoRI sites intact. Following adaptor ligation, the ds cDNAs were phosphorylated at the EcoRI sites and size-fractionated using column to remove small (0.5kb) cDNA fragments and non-cDNA contaminants (0.1kb) (unincorporated primers and unligated adaptors). The resulting cDNA was then cloned into λgt11, which is an EcoRI-digested and phosphorylated phage vector.

We quantified the three test ligations, compared the titers and determined the optimal ratio of vector to cDNA insert. Phage packaging reaction was performed according to the λ-DNA *in vitro* packing module instructions (Amersham LIFE SCIENCE, Cat.#RPN1717). In this procedure, we used cell extracts derived from two induced lysogens whose prophages carry different, but complementing, mutations in the genes required for assembly of mature phage particles. Subsequently, we mixed these cell extracts together with λ-DNA, the DNA was packaged into infectious phage particles and then introduced into *Ecoli* host cells by infection processes. From the five ligations combined, we obtained the unamplified library that contains 1×10^6 independent clones. This library was then amplified, and the titer of amplified library was determined to be 1×10^{10} pfu/ml.

The quality of the cDNA library was checked by PCR for the size of inserts (Figs. 1 and 2) and by endonuclease restriction enzyme analysis. Size-fractionation of synthesized cDNAs showed the peak size to lie in the range of 600 to 2,000 bp. Using PCR and endonuclease restriction enzyme digestion, we determined the average length of our cDNAs to be approximately 1.3 kb.

Conversion of a λ-phage library into a Plasmid cDNA expression library

To generate a plasmid cDNA expression library, there are two important issues to be addressed: 1) an optimal plasmid that allows simultaneous expression of both the gene of interest and a selective marker, 2) a plasmid that contains the tetracyclin-regulated expression systems. Therefore, we used pBI-EGFP (CLONTECH Laboratories, Inc., Cat#6154-1) which is a responsive plasmid that can coexpress a gene of interest and enhanced green fluorescent protein (EGFP) from a bidirectional tetracyclin-responsive promoter (21,22). This pBI-EGFP Tet vector contains the bidirectional promoter that is responsive to the tTA and rtTA regulatory proteins in the Tet-Off and Tet-On Systems, respectively. Furthermore, EGFP is a unique protein that is the brightest known GFP variant and can be expressed in mammalian cells. Importantly, the expression of EGFP

can be visually monitored using fluorescence microscopy as well as Flow Cytometry. Thus, the EGFP reporter gene system provided a very convenient way to detect gene expression and localize the fusion protein within the cells without a specific assay. Consequently, by adapting this excellent system, the time applied on selection of growth inhibited cell lines has been significantly reduced and the quality of plasmid cDNA library transfection has also been greatly improved.

The established λ phage cDNA library was used to construct a plasmid cDNA expression library. The cDNAs were obtained by digesting λ phage cDNA library with the EcoRI restriction enzyme. These cDNAs were then trimmed using Klenow enzymatic reaction to generate blunt-ends suitable for subsequent ligations. Meanwhile, pBI-EGFP was linearized by Pvu II restriction enzyme digestion and dephosphorylated by calf intestinal alkaline phosphatase reaction, respectively. It is important to mention that the quantity and the high quality of the ligations were required to successfully reconstruct this pBI-EGFP cDNA library. Thus, the blunt-end ligations we have performed in this experiment were extremely difficult. In order to accomplish this task, one of the approaches we took was to use the Takara ligase kit (Takara Shuzo Co., Ltd., Cat#6021) which has been shown to be very effective in blunt end ligation (34). While it took considerable effort, we also were able to optimize the ligation conditions. Consequently, the ligation efficiency was greatly improved, and the blunt-end ligations were successfully carried out by integrating cDNAs into linearized pBI-EGFP with the assistance of Takara ligase. pBI-EGFP vectors containing the cDNA library were then introduced into *E. coli* host cells by transformation. With the twenty combined ligations, an unamplified pBI-EGFP cDNA expression library was finally generated, which contains 1×10^6 independent clones. This library was further amplified to be approximately 1×10^{10} pfu/ml. The cDNA library was then purified and ready for transfection.

Establishment of stable LCC6$^{tetR+neoR}$ and MDA231$^{tetR+neoR}$ cell lines

(1) MDA435/LCC6 cells and MDA-MB-231 cells were co-transfected with plasmid expressing the tetR repressor and the G418 resistance marker as described previously (19, 21).

(2) Transfected MDA435/LCC6 and MDA-MB-231 cells were treated with G418 (400 ng/ml).

(3) Surviving LCC6 and MDA231 cells were transfected with pBI-EGFP and regulated with the addition of doxycycline. The selection of Lcc6$^{tetR+neoR}$ and 231$^{tetR+neoR}$ cell lines was performed using fluorescence microscopy.

(4) The selected stable LCC6$^{tetR+neoR}$ and 231$^{tetR+neoR}$ cell lines were further confirmed by transfecting with a plasmid that contains a doxycycline regulable element and expresses luciferase (Promega, Cat# 1500) and assaying the enzymatic activity. Clones with the lowest background and highest induction of expression were selected.

Transfection of LCC6$^{tetR+neoR}$, 231$^{tetR+neoR}$ and MCF-7$^{tetR+neoR}$ cells

To obtain the optimal transfection, there are several basic elements involved: 1) highly efficient transfection reagent, 2) ratio of transfection reagent to plasmid DNA, 3) the quality of plasmid DNA, 4) quantity of transfection complex, and 5) optimal cell confluency, 6) appropriate transfection time (30). Therefore, we chose to use SuperFect transfection reagent (QIAGEN, Cat#301305) which is a specifically designed polycation and showed higher transfection efficiencies than those obtained with many liposome transfection reagents. The ratio of SuperFect and cDNA plasmid was optimized to 4 μl of SuperFect per 1 μg of DNA. Finally, the stable transfection condition was established by transfecting LCC6$^{tetR+neoR}$, MDA231$^{tetR+neoR}$ and MCF-7$^{tetR+neoR}$ cells at 30% confluence in T-75 flask with a complex of 40 μg of DNA and 160 μl of SuperFect for a 95-minute-incubation period. This resulted in maximum transfection efficiency (about 40-50%) and minimum cytotoxic effects. Transfection efficiency was further verified by transfecting LCC6$^{tetR+neoR}$, MDA231$^{tetR+neoR}$ and MCF-7$^{tetR+neoR}$ cells with a plasmid expressing β-galactosidase, which was assayed colorimetrically using the substrate *O*-nitrophenyl-β-D-galactopyranoside.

Identification of cells containing growth inhibitory genes

To help identify growth inhibited cells, cells were labeled with PKH26-GL (Sigma Chemical Co. St Louis, MO, Cat# 17621), a non-toxic, red fluorescent cell linker that is incorporated into the cell membrane by selective partitioning (24). Importantly, it is retained in non-proliferating cells. The appearance of labeled cells varies from bright and uniform labeling to punctate and patch appearance. However, one problem is an over-labeling that can result in loss of membrane integrity and cell recovery. Thus, it was essential to determine the best ratio of dye to cell. In our experiment, the optimal concentration of dye and cell was determined to be approximately 2 x 10^{-6} M PKH26-GL and 1 x10^{7} cells/ml. The PKH26-GL labeled LCC6, 231, and MCF-7 cells were then checked for their recovery, viability, and their fluorescence intensity using fluorescence microscopy and Flow Cytometry.

To enrich bulk transfected cell populations for growth inhibited cells, a systemic approach has been developed in this study. MCF-7 cells already transfected with a plasmid expressing both the tetR repressor and the G418 resistance marker were initially treated with G418. Surviving cell populations were then stained with PKH26-GL and grown for 24-36 hours. PKH26-GL labeled MCF-7 cells were subsequently transfected

with pBI-EGFP cDNA expression library using SuperFect and optimal transfection conditions as described previously. pBI-EGFP cDNA vectors have each of the transfected cDNAs expressed under the control of the tetracycline resistance operon that is negatively regulated by doxycycline. This system also enabled us to establish the double stable cell lines.

Cells transfected with pBI-EGFP cDNA library were grown, in the absence of tet, for the equivalent of several generations and then selected for growth suppressed clones. The identification of those cell clones that contained growth inhibiting cDNAs was performed by selecting cells that concurrently exhibit EGFP expression and strong PKH26-GL labeling. Therefore, single EGFP expressing (green) and PKH26-GL (red) double-labeled cells were aseptically sorted by FACS into the wells of 96-well plates after 96-120 hours of transfection.

To date, we have single-cell sorted fourteen 96-well plates, which provided a significant number of individual cell clones expressing growth inhibitory genes. Immediately after sorting, doxycycline was added to the culture medium at a concentration of 1 μg/ml, and cell clones containing growth suppressing cDNAs were rapidly expanded by releasing the growth suppression. From these plates, we have obtained 22 single cell sorted populations that meet the selection criteria and were growth suppressed upon withdrawal of doxycycline. These were expanded and frozen for future analyses.

Technical Objective 2: Characterize putative TSG(s)

Task 5: cDNA sequencing and sequence analysis.

Task 6: Screen tumors for mutations in putative TSG(s).

Cloning growth suppressing genes

To clone growth-inhibiting genes, identified cell clones containing growth suppressing genes were quickly enriched from single cell to multiple cells with the addition of tet. Of the utmost importance to succeed this experiment is to avoid the contamination during cell population enrichment. Single cells were first sorted into the medium containing doxycycline at the concentration of 1 μg/ml and 1x Penicillin Streptomycin (GibcoBRL, Cat# 5140-122). Twenty-four hours after sorting, the medium was changed with the addition of anti-fungal and yeast agents, Amphotericin B (Sigma, Cat# A-2940) and Nystatin (Sigma, Cat# N-4014) with the concentrations commercially suggested. The next day, the medium was changed back with only 1 μg/ml of doxycycline and 1 x Penicillin Streptomycin. Since then, the medium was replaced almost every 24 hours, but

every 5 days added with Amphotericin B and Nystatin. During this selection process, cells were gradually transferred from 96-well plates into 48-well plates, 24-well plates, 12-well plated, 6-well plates, T-25 flasks and T-75 flasks. The further selection was performed with G418 (400 μg/ml). The selected cell clones were stored in −70°C and then liquid nitrogen.

The putative growth inhibiting genes were identified by PCR with either TaqBead™ Hot Start Polymerase (Promega, Cat#M5661), or Expand™ High Fidelity PCR System (Boehringer Mannheim, Cat#1 732 650) using the genomic DNAs as templates, which were extracted from the cell clones containing growth suppressing genes. The PCR primers were designed containing partial sequences of pBI-EGFP, one pair # 426 (5'-GTACCCGGGTCGAGTAGGCGTGTA-3') and # 650 (5'-GGTCCCCAAACTCACCCTGAAGT-3'), and another pair # 426 and # 657 (CAATCAAGGGTCCCCAAACTCACC-3'), according to primer design programs (DNAStar). Several PCR products were found and ranged from 600 bp to 2 kb. The PCR segments were cut out from the gels and purified. The purified cDNAs were then re-amplified with the same primers. The obtained cDNA products were sequenced either by direct sequencing or subcloning the cDNAs into the vector and then sequencing.

Sequencing of two putative cDNAs cloned from growth inhibited clones

Using the methods described above we isolated two putative cDNAs from MDA231 transfected cells. The clones were sequenced and the sequences run against the NCBI databases to identify possible origins. One sequence is vector DNA but the second sequence is highly homologous to a sequence of human chromosome #9 (sequence included in the appendix.). Our sequence is too short to estimate experimentally its likely location on chromosome #9. However, the sequence homology search indicates that it occurs on chromosome #9p, relatively close to the beginning of the chromosome. Several areas of LOH on chromosome #9 have been reported in breast tumors. However, our sequence is probably not involved in the 9p23-p21 LOH associated with BRCA2 (36). Several other sites of LOH, which are associated with tumor suppressor gene activity, are thought to occur on chromosome #9 (37).

6. Key Research Accomplishments.

- Generated high quality cDNA library from reduction mammoplasty.
- Expressed cDNA library in human breast cancer cells.
- Identified growth inhibited single celled clones and stored cloned cells.
- Cloned cDNAs from growth inhibited cell clones.
- Sequenced and identified two putative cDNAs, one of which appears to be located on human chromosome #9.
- Successfully completed training of one Postdoctoral Fellow (Dr. Pu) and begun training of another (Dr. Zhu).

7. Reportable Outcomes

i) Pu, L.P., Skaar, T.C., Gu, Z.P., Leonessa, F. **& Clarke, R.** "A novel selection system for identifying growth suppressed human breast cells." *Proc Am Assoc Cancer Res* 40: 32, 1999.

ii) Pu, L.-P., Skaar, T.C., Leonessa, F. **& Clarke, R.** "Tumor suppressor genes in breast cancer". *DOD Breast Cancer Research Program* pp108, 2000.

iii) Developed a series of clones of human breast cancer cells containing putative TSGs.

iv) Dr. Pu successfully entered a Clinical Residency Program at the University of Cincinnati.

8. Conclusions

While we have not definitively identified a new tumor suppressor gene, we have multiple cell line clones from which to identify candidates. We consider it encouraging that our first putative clone is on Chromosome #9, a chromosome where LOH has been observed in some breast cancers. Clearly additional studies are necessary to assess the likely relevance of this observation. We are somewhat disappointed that we did not find either a known TSG, or homology to a known gene(s) in the chromosome #9 sequence, either of which would have made publication of the method feasible. However, we hope to obtain

additional data that will result in at least one full publication. We have two published abstracts, both of which were well received when presented.

This is a postdoctoral fellowship application. Most of the work was completed by an individual who previously worked in another field, and was not the original recipient of the award (Dr. Pu). The project was held back due to this candidate having another child, and later successfully competing for a clinical residency. The remaining effort was provided by a third Fellow (Dr. Zhu). Nonetheless, we have managed to complete key aspects of the proposed work, despite these losses of time and the high-risk nature of the study. Thus, we believe that the major goals of the application, in terms of training and research, have been met.

9. References.

1. van de Vijver, M.J. Molecular genetic changes in human breast cancer. Adv. Cancer Res. 61:25-56, 1994.
2. Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A., McGuire, W.L.Science. 235:177-182, 1987.
3. Devilee, P., Cornelisse, C.J. Somatic genetic changes in human breast cancer. Biochim Biophys Acta. 1198:113-30, 1994.
4. Elledge R.M., Allred, D.C. The p53 tumor suppressor gene in breast cancer. Breast Cancer Res. Treat. 32:39-47, 1994.
5. Garkavtsev, I., Grigorian I.A., Ossovskaya, V.S., Chernov, M.V., Chumakov, P.M., Gudkov A.V. The candidate tumour suppressor p33 ^{ING1} cooperates with p53 in cell growth control. Nature 391:295-298, 1998.
6. Greenblatt M.S., Bennett W.P., Hollstein M., Harris C.C. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. (review). Cancer Res. 54:4855-78, 1994.
7. Knudson, A.G., Jr. A two-mutation model for human cancer. In: Advances in viral oncology, Vol. 7. G. Klein Ed. Raven Press, New York, 1987, pp.1-17.
8. Xiao S., Li D., Corson J.M., Vijg J., and Fletcher J.A. Codeletion of p15 and p16 genes in primary non-small cell lung carcinoma. Cancer Res. 55:2968-71, 1995.
9. Toyooka M., Konishi M., Kikuchi-Yanoshita R., Iwama T., and Miyaki M. Somatic mutations of the adenomatous polyposis coli gene in gastroduodenal tumors from patients with familial adenomatous polyposis. Cancer Res. 55:3165-70, 1995.
10. Zhou X., Tarmin L., Yin J., Jiang H-Y., Suzuki H., Rhyu M-G., Abraham J.M., Meltzer tS.J. The MTS1 gene is frequently mutated in primary human esophageal tumors. Oncogene. 9:3737-41, 1994.

11. Suzuki H., Zhou X., Yin J., Lei J-Y., Jiang H-Y., Suzuki Y., Chan T., Hannon G.J., Mergner W.J., Abraham J.M., Meltzer S.J. Intragenic mutations of CDKN2B and CDKN2A in primary human esophageal cancers. Human Mol. Genet. 4:1883-1887, 1995.
12. Viskochil, D., Buchberg, A.M., Xu, G., Cawthon, R.M., Stevens, J., Wolff, R.K., Culver, M., Carey, J.C., Copeland, N.G., Jenkins, N.A., White, R., O'Connell, P. Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type 1 locus, Cell 62:187-92, 1990.
13. Culotta E., and Koshland D.E., Jr. p53 sweeps through cancer research. Science. 262:1958-61, 1993.
14. Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P.A., Skolnick, M.H., et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA 1. Science. 266:66-71, 1994
15. Futreal, P.A., Liu, Q., Shattuck-Eidens, D., Cochran, C., Harshman, K., Tavtigian, S., et al. BRCA 1 mutations in primary breast and ovarian carcinomas. Science. 266:120-2, 1994.
16. Huusko P., Paakkonen K., Launonen V., Poyhonen M., Blanco G., Kauppila A., Puistola U., Kiviniemi H., Kujala M., Leisti J., Winqvist R. Evidence of founder mutations in Finnish BRCA1 and BRCA2 families. Am J Hum Genet. 62:1544-1548, 1998.
17. Blackwood M.A., Weber B.L. BRCA1 and BRCA2: from molecular genetics to clinical medicine. J Clin Oncol 16:1969-1977, 1998.
18. Gossen M., Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci U S A. 89:5547-5551, 1992.
19. Gossen M., Freundlieb S., Bender G., Muller G., Hillen W., Bujard H. Transcriptional activation by tetracyclines in mammalian cells. Science 268:1766-1769, 1995.
20. Baron U., Freundlieb S., Gossen M., Bujard H. Co-regulation of two gene activities by tetracycline via a bidirectional promoter. Nucleic Acids Res 23:3605-3606, 1995.
21. Cormack B.P., Valdivia R.H., Falkow S. FACS-optimized mutants of the green fluorescent protein (GFP). Gene 173:33-38, 1996.
22. Maines J.Z., Sunnarborg A., Rogers L.M., Mandavilli A., Spielmann R., Boyd F.T. Positive selection of growth-inhibitory genes. Cell Growth Differ 6:665-671 1995.
23. Horan P.K., Slezak S.E. Stable cell membrane labelling. Nature 340:167-168, 1989.
24. Clarke R., Brunner N., Katz D., Glanz P., Dickson R.B., Lippman M.E., Kern F.G. The effects of a constitutive expression of transforming growth factor-alpha on the growth of MCF-7 human breast cancer cells in vitro and in vivo. Mol Endocrinol 3:372-380, 1989.
25. Clarke R., Brunner N., Katzenellenbogen B.S., Thompson E.W., Norman M.J., Koppi C., Paik S., Lippman M.E., Dickson R.B. Progression of human breast cancer cells from hormone-dependent to hormone-independent growth both in vitro and in vivo. Proc Natl Acad Sci U S 86:3649-3653, 1989.

26. Clarke R., Brunner N., Thompson E.W., Glanz P., Katz D., Dickson R.B., Lippman M.E. The inter-relationships between ovarian-independent growth, tumorigenicity, invasiveness and antioestrogen resistance in the malignant progression of human breast cancer. J Endocrinol 122:331-340, 1989.
27. Brunner N., Frandsen T.L., Holst-Hansen C., Bei M., Thompson E.W., Wakeling A.E., Lippman M.E., Clarke R. MCF7/LCC2: a 4-hydroxytamoxifen resistant human breast cancer variant that retains sensitivity to the steroidal antiestrogen ICI 182,780. Cancer Res 53:3229-3232, 1993.
28. Brunner N., Boulay V., Fojo A., Freter C.E., Lippman M.E., Clarke R. Acquisition of hormone-independent growth in MCF-7 cells is accompanied by increased expression of estrogen-regulated genes but without detectable DNA amplifications. Cancer Res 53:283-90, 1993.
29. Sambrook, J. *et al.* Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 1989. 30. Fromont-Racine M., Bertrand E., Pictet R., Grange T. A highly sensitive method for mapping the 5' termini of mRNAs. Nucleic Acids Res 21:1683-1684, 1993.
31. Chenchik A., Diachenko L., Moqadam F., Tarabykin V., Lukyanov S., Siebert P.D. Full-length cDNA cloning and determination of mRNA 5' and 3' ends by amplification of adaptor-ligated cDNA. Biotechniques 21:526-534, 1996.
32. Barnes W.M. PCR amplification of up to 35-kb DNA with high fidelity and high yield from lambda bacteriophage templates. Proc Natl Acad Sci U S A 91:2216-20, 1994
33. Hayashi K., Nakazawa M., Ishizaki Y., Hiraoka N., Obayashi A. Regulation of inter- and intramolecular ligation with T4 DNA ligase in the presence of polyethylene glycol. Nucleic Acids Res 14:7617-7631, 1986.
34. Liang P., Pardee A.B. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257:967-971, 1992.
35. Soule H.D., Maloney T.M., Wolman S.R., Peterson W.D. Jr., Brenz R., McGrath C.M., Russo J., Pauley R.J., Jones R.F., Brooks S.C. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res 50:6075-86, 1990.
36. Savelyeva L., Claas A., Matzner I., Schlag P., Hofmann W., Scherneck S,. Weber B., Schwab M. Constitutional genomic instability with inversions, duplications, and amplifications in 9p23-24 in BRCA2 mutation carriers. Cancer Res 61: 5179-5185, 2001.
37. Marsh K.L., Varley J.M. Loss of heterozygosity at chromosome 9p in ductal carcinoma in situ and invasive carcinoma of the breast. Br J Cancer 77: 1439-1447, 1998.

10. Appendices

DNA sequences, putative cDNA and protein structures and homologies.

Figures and figure legends.

Figure 1. PCR screening inserts in λgt11 cDNA library. Lambda DNA was prepared by picking up a plaque with a micropipette and transferring into deionized H_2O. PCR was performed basically according to CLONTECH's LD-Insert Screening Amplimer Sets (CLONTECH Laboratories, Inc., Cat # PT1579-1). This PCR showed different size of inserts. Lanes 1-4 used 5% DMSO, and lanes 5-7 used 10% DMSO. Note the arrow that the size of insert (lane 5) is approximately 1 kb.

Figure 2. PCR screening the size of inserts in λgt11 cDNA library. Lambda DNA was prepared from Midi-Prep DNA extraction. This PCR showed the peak size to lie in the range of 600 to 2,000 bp.

Figure 3. Selection of cell populations containing growth suppressing genes. Cell clones stained with PKH26-GL and transfected with pBI-EGFP cDNA expression library were grown, in the absence of tet, identified by cells that, simultaneously, expressed enhanced green fluorescent protein (green) and had brightest PKH26-GL labeling (red) and then sorted by FACS into 96-well plates. Cells (square 2) were selected as green and red double fluorescence expressing cell. However, cells were considered as either single red (square 1) and green (square 4) or no (square 3) fluorescence labeling.

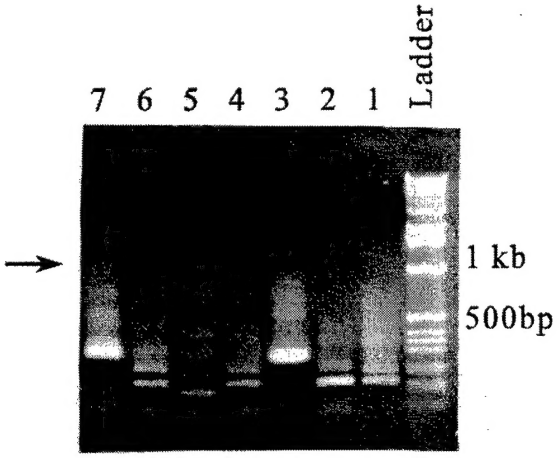


Fig 1: PCR screening inserts in λgt11 cDNA library. size of inserts in λgt11

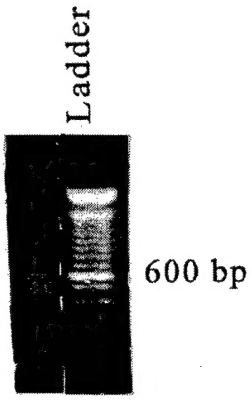


Fig 2: PCR screening the cDNA library.

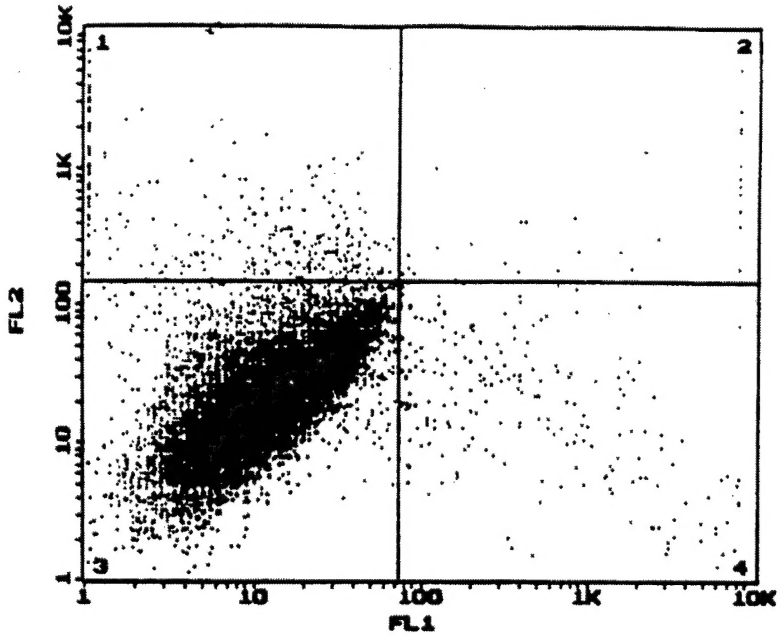
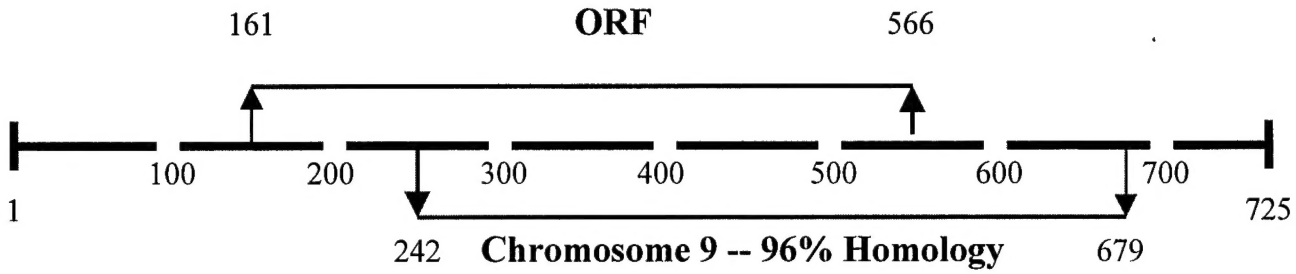


Fig 3: Selection of cell populations containing growth suppressing genes.



We found 96% homology to a genomic sequence on chromosome #9 from bp242-679 of our putative cDNA. We could find no meaningful homology for bp1-241 or 680-725. These observations are potentially consistent with a cDNA of at least three exons, encoded by a gene on chromosome #9. We searched the sequence in all three frames for putative open reading frames (ORF). We found one putative ORF: Predicted Open Reading Frame (ORF = bp161 to bp566). This is a rather tentative prediction, since some bases could not be fully identified during sequencing and we were willing to tolerate up to one possible stop codon within the ORF.

Using other algorithms in this software package, we obtained a putative amino acid sequence, implicating a protein with MW = 14.5 kDa. The predicted physical characteristics of the protein, if translated from the cDNA Sequence (bp161 to bp566) with Standard Genetic Code, were generated by the DNAStar analysis software:-

Molecular Weight 14529.37 Daltons (approx 14.5 kDa)
132 Amino Acids
10 Strongly Basic (+) Amino Acids (K,R)
2 Strongly Acidic (-) Amino Acids (D,E)
35 Hydrophobic Amino Acids (A,I,L,F,W,V)
24 Polar Amino Acids (N,C,Q,S,T,Y)

11.041 Isoelectric Point
9.207 Charge at PH 7.0

Total number of bases translated = 405

% A = 21.23	[n=86]
% G = 24.20	[n=98]
% T = 17.28	[n=70]
% C = 30.12	[n=122]
% Ambiguous = 7.16	[n=29]
% A+T = 38.52	[n=156]
% C+G = 54.32	[n=220]

This putative protein is predicted to exhibit several additional characteristics. The protein may be a substrate for a serine-threonine kinase, since NetPhos (vs. 2.0) predicts two serine phosphorylation sites at amino acids 86 and 119, and a threonine phosphorylation site at amino acid 37. If translated, the protein may be subject to further post-translational modification by N-glycosylation at amino acid 103 and N-myristolation at amino acids 27-32 and 36-41 (Motif Scan algorithm).

Nucleic acid sequence

1
NGTGGNNTGNNNNNTNNNNNNTTCCTTATGGGGACCGNNTTNTANGAACTCT
ATNGGCCCACAGGATCTCNATGANNCACCACTAGGTAACCNCNTNGNATCAG
GNGCTGGGGGTGANANGTCGGTCACGGTCTNTTCCAAACNNGNTNNNNNAN
160

161
ATGGTGTCGNTTCNNCANCTTTGGGTNTNANAGNGGNTCTCNNNCAATNAAT
NACTCTGANCCTTGNNGNCTAANNAACNNGGAGGAACCGANACCTGANCCC
CCACCCGTGGGACAGGCCCTGGGCACTANGGAAAGGCCCCANGAGCCCCTCC
AATTGGCCACCTGGCCTCAGATGCCCTGCGTTACCACAGAGGCCTCCAGCAG
TTGTCTATCTGGGTCTCAGCTGAGGTGCCCACAGCTACAAGATCAGGACAGA
GATCACAGGGGAGAGTGGCCCACCAGGCAGGGAGATGGAAACCAGGTCCTC
ACCATCTNTGCCACTAACTGCCCCCTGCATACCCTTGGGCCAAGCCTGCG
CCCTCTGTGCCTGGACTACCCTACCTGCANGGTATANAGCACATGA
566

567
CCTGCCAAGGAGTGTGGCTGCTTGGCCACTGCCCTAAGTTCTTGCCTCCGCTA
NCCTACCAACTCTGGCCTGTTAACCTGACTACCCACTTCCCTGANCANACACA
TACACGCCTACTCNNNNNNCCCCGNGNNANNNNNNNNNNNNNNNN
NTNNTTN
725

N=not determined

Predicted amino acid sequence for ORF= bp161 to bp566
MVSXXXLWVXXXXSXNXXLXLXXXXXGGTXTXPTRGTGPGHXGKAPXAPPIG
HLASDALRYHRGLQQLSIWVSAEVPTATRSGQRSQGRVAHQAGRWKPGPHHLC
HLPPAYPWAKPAPSVPGLPYLXGIXH

X=uncertain designation (probably due to undetermined nucleic acid).